Cy5.5-Annexin V

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Chemical name:	Cy5.5-Annexin V
Abbreviated name:	Cy5.5-Annexin V
Synonym:	
Backbone:	Protein
Target:	Phosphatidylserine
Mechanism:	Binding
Method of detection:	Optical, near-infrared fluorescence
Source of signal:	Cy5.5
Activation:	No
***In vitro* studies:**	Yes
Rodent studies:	Yes
Other non-primate mammal studies:	No
Non-human primate studies:	No
Human studies:	No

Background

[PubMed]

Apoptosis, or programmed cell death, is a fundamental component of tissue development and differentiation and plays an important role in a great variety of diseases, including cancer. Excessive apoptosis is observed in diseases such as AIDS, Alzheimer's, and progressive heart failure, whereas insufficient apoptosis often occurs in tumor growth. It also plays a central role in the pathology of type 1 and type 2 diabetes (1). Imaging of apoptosis may be a valuable tool for quantifying the development (or regression) of such diseases and for monitoring the effects of chemotherapies (2), anti-hormonal therapeutics, and anti-angiogenic therapies.

The highly regulated mechanism of cell apoptosis involves an externalization process of aminophospholipids, primarily phosphatidylserine (PS), that normally face the cytoplasm. Through this process, PS residues are exposed at the outer plasma membrane and face the extracellular fluid. At this stage, Annexin V (36 kDa; $10^{-10} < K_d < 10^{-9}$ M (3)) may be used to recognize the surface-exposed PS by strongly and specifically binding to the phospholipid, reflecting a biological role as an anti-coagulant (4-6).

Imaging of apoptosis through PS exposure using Annexin V removes the need for cellular internalization. This *in vivo* imaging method, which has increased in popularity in recent years, has led to the development of both fluorescent and magnetic annexins for various applications (7-9). Tagging Annexin V with a fluorophore produces a probe that acts similarly to native Annexin V and preserves the mass, high affinity, and specificity of the protein.

Nevertheless, Annexin V can be retained in normal tissues and interfere with the imaging of apoptotic uptake. Because the changes in signal are usually small in the case of apoptosis, the sensitivity of the imaging technique is therefore an important parameter to consider (10). Several studies have also shown the ability of Annexin V to bind to PS exposed on the cell membrane in other pathological conditions, such as widespread cutaneous necrosis resulting from vascular damage (e.g., antiphospholipid antibody (aPL) syndrome associated with dermal microvascular thrombosis) (11, 12).

Cy5.5 is a fluorophore with low hydrophobicity and high photostability that can be used as a probe for near-infrared fluorescence (NIRF) imaging of tumor apoptosis using Annexin V (13). Its absorbance and emission maxima are 683 nm and 707 nm respectively (measured in 0.1 M triethylammonium acetate (TEAA) buffer, pH 7).

Synthesis

[PubMed]

Annexin V may be purified from human placenta. The general procedure involves the preparation of recombinant Annexin V by cytoplasmic expression of *Escherichia coli* strain BL21(DE3) containing plasmid pET2a-PAPI, following a procedure described by Wood et al. (14). In brief, a culture of the Annexin V cell line is grown to saturation; the cells are then sonicated in 50 mM Tris-HCl at pH 7.2, with10 mM $CaCl_2$, and centrifuged for 10 min at 2500 × *g* at 4°C. The Annexin V bound to bacterial membranes is removed by resuspending the pellet in 50 mM Tris-HCl at pH 7.2, with 20 mM EDTA. The supernatant is then dialyzed against 50 mM HEPES at pH 7.4. The final yield obtained by following this procedure is about 45 mg/ of culture, with a purity of 98-99% (values obtained using electrophoresis with a polyacrylamide gel). A comparable method for the preparation of Annexin V is given by Schellenberger et al. (15).

The synthesis of Cy5.5-Annexin V can be obtained by modification of Annexin V with the *N*-hydroxysuccinimide ester of Cy at two different ratios of Cy:Annexin V (2 mg/mg and 20 mg/mg) and an inactive Annexin V (approximately an equimolar ratio of Cy:Annexin) (7).

The labeling of Annexin V with Cy5.5 can be done by activating Annexin V (0.3 ml, 3 mg/ml in 0.1 bicarbonate, pH 8) with a solution containing Cy5.5 monohydroxysuccinimide ester (about 2 mg in 40 µl of dimethyl sulfoxide), as described by Petrovsky et al. (7). After incubation of the reaction mixture for 1 h at room temperature, the covalent conjugate of Cy5.5-Annexin V is separated from non-reacted Cy5.5 by double spin separation on BioGel P6 in phosphate-buffered saline at pH 7.4. Fluorescence quenching may be assessed after treatment of Annexin V with trypsin (100 µg/ml, 2 h at 37ºC). Using this procedure, active Cy-Annexin V had, on average, 1.1 Cy molecules bound per mol of Annexin V. To synthesize inactive Cy-Annexin (>2 mol of Cy/mol protein), a 10 times higher ratio of Annexin V was used to get a higher degree of amino-group modification (7).

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

The common method for assessing the ability of Annexin V (and Cy5.5-Annexin V) to differentiate apoptotic and normal cells is to induce apoptosis in Jurkat T-cell lymphoma cells, as described in the published literature (7, 13, 15). Wood et al (14). used camptothecin-treated cells and quantified the biological cell affinity of Cy5.5-Annexin V with a red diode laser (635 nm) and > 670-nm bandpass filter (FL4 channel). Cytometry of treated (apoptotic) cells and untreated cells (incubated using active Annexin V) showed two distinct populations. NIRF was 6-10 times higher for apoptotic cells than for live cells.

Medarova et al. (1) assessed the potential of Cy5.5-Annexin V as a NIRF probe for diabetes-associated β-cell apoptosis in mouse models of type 1 and type 2 diabetes. Both *in vitro* and *ex vivo* studies were performed using mice of different ages. Four models were used: the MDLS model (type 1 diabetes), the SHDS model (type 1 diabetes), the NOD mouse model (type 1 diabetes, symptoms age-dependent), and the *db/db* model (type 2 diabetes). Mice were treated with streptozotocin (STZ) for type 1 diabetes models. Cy5.5-Annexin V was injected intravenously, and *ex vivo* NIRF imaging was performed 6 h after injection on snap-frozen pancreatic sections from euthanized animals.

In the case of the SHDS model, NIRF showed a selective accumulation of Cy5.5-Annexin V in the pancreata of STZ-induced diabetic mice, which was confirmed by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay on frozen sections (26.3 ± 0.8 and 17.5 ± 0.63%, respectively). In the MLDS model, only a few cells within islets bound Annexin V (2.3 ± 0.2 and 1.6 ± 0.2%, respectively). In the case of the NOD model, 3- and 6-week-old mice showed no binding to Cy5.5-Annexin V, whereas in 8- and 12-week-old mice, 2.6 ± 0.3 and 3.2 ± 0.2% of nuclei were found to specifically bind to Cy5.5-Annexin V. Apoptotic rates by TUNEL staining were found to be 0.6 ± 0.1, 1.2 ± 0.2, and 2.3 ± 0.3%, respectively. In the case of *db/db* mice (type 2 diabetes), the apoptotic rate of pancreatic tissue sections determined by Cy5.5-Annexin V was found to be 2.3 ± 0.3%, and 2.0 ± 0.1% by TUNEL staining.

Animal Studies

Rodents

[PubMed]

Rodent studies were performed on *nu/nu* female mice bearing green fluorescent protein (GFP)-expressing tumors, following the published procedure (7). GFP fluorescence clearly defined the outline of the tumors, which helped to identify the NIRF signal from the tumors and the signal obtained from normal tissue.

Cy5.5-Annexin V was injected intravenously (i.v.) via the tail (3.1 mg of Cy5.5-Annexin V/kg). A weak NIRF signal was obtained 90 min after injection from tumor and non-tumor tissues. Animals were then treated with a single injection of cyclophosphamide (CPA) (170 mg/kg), and active Cy5.5-Annexin V was re-injected after a 24-h period. NIRF imaging was then performed. In contrast to non-treated animals, CPA-treated mice showed a strong enhancement of the tumor NIRF signal at 75 min after injection, and the signal was 2-3-fold higher for CPA-treated tumors than non-treated ones ($p < 0.05$). Histology analysis, following the protocol described by Petrovsky et al. (7), showed

that the density of cells with active Cy5.5-Annexin V was only 1-2 cells/mm^2 for non-CPA-treated tumors and 17 ± 4 cells/mm^2 for CPA-treated tumors. In both cases, Cy5.5-Annexin V binding cells were found to be a very small fraction of the total number of cells present.

Additional studies were performed by Petrovsky et al. (7) using an *in vivo* cancer model with a CPA-resistant variant of Lewis lung carcinoma (Ds Red2 CR8). In this case, biodistribution studies after a 90-min injection of Cy5.5-Annexin V showed that the tracer was bound to tumors of CPA-treated animals at 12.8 ± 4.9% of the injected dose/g of tissue, whereas control tumors accumulated 4.4 ± 1.6% of the injected dose/g of tissue. Cy5.5-Annexin V was found to bind to the kidney (a major site of annexin accumulation) in a similar manner for both treated animals (6.9 ± 2.6 injected dose/g) and control groups (8.5 ± 2.6 injected dose/g).

Other Non-Primate Mammals

[PubMed]

No publication is currently available.

Non-Human Primates

[PubMed]

No publication is currently available.

Human Studies

[PubMed]

No publication is currently available.

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